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RESEARCH REPORT

Quick Diagnosis of Alkaptonuria by Homogentisic Acid Determination in Urine Paper Spots

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Abstract *Objectives*: Two methods are described for homogentisic acid (HGA) determination in dried urine spots (DUS) on paper from Alkaptonuria (AKU) patients, devised for quick early diagnosis. AKU is a rare autosomal recessive disorder caused by deficiency of homogentisate 1,2-dioxygenase, yielding in accumulation of HGA. Its massive excretion causes urine darkening by exposure to air or alkalinization, and is a diagnostic marker. The deposition of polymers produced after HGA oxidation within the connective tissues causes ochronotic arthritis, a degenerative joint disease manifesting in adulthood and only rarely in childhood. No early diagnosis is usually accomplished, awareness following symptom development.

Design and methods: Two methods were designed for HGA determination in DUS: (1) a rapid semi-quantitative reliable method based on colour development in alkali and quantification by comparison with dried paper spots from HGA solutions of known concentration and (2) a quantitative and sensitive HPLC-linked method, previously devised for purine and pyrimidine analysis in urine and plasma.

Results: Colour intensity developed by DUS after alkali addition was proportional to HGA concentration, and calculated amounts were in good agreement with quantitative analysis performed by RP-HPLC on DUS and on urines as such.

Conclusions: DUS, often used for different diagnostic purpose, are easily prepared and safely delivered. The simple and quick colour method proposed provides reliable HGA assessment and is fit for large screening. HGA concentration determined in 10 AKU patient DUS by both methods 1 and 2 was in agreement with direct urine assay and in the range reported by literature.

A reliable HGA quantification based on colour development in paper urine spots is validated by HPLC-linked HGA quantification, and proposed as a quick diagnostic tool for AKU patients.

Introduction

Alkaptonuria (AKU, OMIM: 203500) is a rare disease, associated with inherited recessive mutations in *HGD* gene leading to homogentisate 1,2-dioxygenase (HGD, E. C.1.13.11.5) deficiency. As a result homogentisic acid (HGA), deriving from tyrosine and phenylalanine, accumulates and is massively excreted in urine, causing its darkening after air exposure. Brown coloured urine is considered a pathognomonic sign of AKU. The deposition of an "ochronotic" pigment produced after circulating HGA oxidation within the articular connective tissues causes ochronotic arthritis, a degenerative joint disease (Ranganath et al. 2013; Millucci et al. 2015); deposition may also occur in cardiac valves, leading to cardiovascular pathologies. Currently, there is no therapy for AKU, but a clinical trial with nitisinone is in progress (Ranganath et al. 2016).

AKU diagnosis may be delayed until the ochronotic arthropathy manifests, usually in adulthood, and very few data are available about childhood and the earlier stages of the disease. Dark colour development in urines can be delayed depending on urine acidity, and misdiagnosis can

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occur. No clear relationship between circulating HGA amount and symptom severity, age, sex, etc., has been described. Early identification of AKU patients would allow epidemiologic studies and important correlations with physiopathologic parameters.

Dried urine spots (DUS) are used in the diagnosis of inherited disorders, included AKU as a sporadic finding, usually as a qualitative diagnostic tool (Bradley 1975; Barbas et al. 2002).

Dark brown colour is well known to develop by adding alkali (e.g. sodium hydroxide) to either HGA solutions or AKU urines (Bradley 1975; Mathieu et al. 1997; Zibolen et al. 2000; Koska and Srsen 1977; Zhao et al. 2009; Pecker et al. 2008).

In this paper two methods for HGA determination in DUS are presented: method 1, a rapid semi-quantitative method based on colour development in alkali, to be used for the early diagnosis of AKU, and method 2, a quantitative reliable HPLC-linked method. The novelty in the proposed method 1 is HGA quantification by a simple reference colour scale quickly providing reliable results. The advantage in method 2 is the accurate HGA quantification in single dried urine spots. Results from 10 AKU patients are presented.

Materials and methods

Chemicals for HPLC separation were of the highest quality available and reagents of analytical grade were purchased from SIGMA (St. Louis MO, USA). The filter paper used to spot urine was S&S® 2992™, Schleicher & Schuell.

Patients

Adult patients (five females and five males ranging 42–69 years, 54.7 ± 10.5 mean ± sd) presenting different degrees of ochronotic arthropathy were studied after the clinical diagnosis of AKU was established. None of the patients was under specific treatment. Age-matched controls of both sexes with no arthropathies or metabolic disorders were also analysed ($n = 28$). Urine samples (single specimen or 24 h collection) were obtained from patients and controls and dried urine spots were prepared.

The whole study was conducted following the approval of Siena University Hospital Ethics Committee. Patients gave a written informed consent prior to inclusion in the study. The informed consent conformed to the standards set by the latest revision of the Declaration of Helsinki containing few substantive changes relative to the April 2013 iteration.

Dried Urine Spot (DUS) Preparation

Urine samples from 10 AKU patients were both analysed as such and used for DUS preparation by dropping 30 µL on filter paper S&S® 2992™ Schleicher & Schuell. Spots were allowed to dry in darkness for at least 3–4 h at room temperature. Spots of HGA solutions from 1.4 to 46 mmol/L or from 2.5 to 40 mmol/L were prepared following the same procedure. DUS and spots of HGA solutions were used both for colour development (method 1) or eluted for HPLC analysis (method 2, described in Sects. 2.4 and 2.5).

Semi-Quantitative Colour-Based Method for DUS (Method 1)

Brown colour was developed dropping 10 µl of 1 mol/L NaOH on dried spots of both patient urine and HGA solutions. Good colour development was also obtained adding 1 mol/L KOH, K_2CO_3, but not NH_4OH (data not shown); NaOH was used routinely.

Semi-quantitative evaluation of urine HGA was achieved by comparison of colour intensity developed by DUS with that developed by HGA solution spots.

HPLC Analysis

A previously described HPLC method, initially devised to detect purines, pyrimidines, and other metabolites in plasma and urines (Micheli et al. 1999), was used to measure HGA in DUS, in HGA solution spots and in urines.

HPLC apparatus was a System Gold module 125S with a mod. 168 diode array detector module. The column was Phenomenex C18 RP (75 mm × 4.6 mm × 3 µm) equipped with Phenomenex Security guard column (4 × 3 mm). Analysis was performed by gradient elution using 10 mmol/L potassium phosphate, pH 5.5 (Eluant A) and methanol (Eluant B). Sample injection volume was 50 µL. The HPLC procedures were performed at room temperature, with 1 mL/min flow rate; absorbance was monitored at 260 and 280 nm. The elution pattern was as follows: isocratic phase at 100% A for 4 min, then to 21% B in 4 min, then immediately to 30% B and back to initial conditions after 3 min; initial conditions were restored in 7 min, total run time was 18 min. Peak identities were confirmed by retention time, coelution with added standards, 280/260 nm absorbance ratios and UV/Vis spectra. All standard solutions (creatinine, uric acid, L-tyrosine, hypoxanthine, xanthine, phenylalanine, tryptophan and HGA) were prepared in deionized water and stored at -20°C. HGA 40–50 mmol/L stock solutions were aliquoted and stored at

−20°C; suitable dilutions were obtained when needed after quick defreezing. Concentration/peak area linear plots were developed for quantification. A minimum of six calibration points, from 0.02 to 0.8 mmol/L, were used for HGA quantification. Linearity was checked using standard curves fitted by linear regression, and performance of fitted curves is presented as the coefficient of determination (r^2).

Accuracy was determined as closeness to the nominal spiked concentration in control urine with $n = 6$. Precision was determined with $n = 6$ and expressed as coefficient of variation (CV). The limit of quantification was the lowest concentration allowing a CV<20%.

Quantitative HPLC-Linked Method for DUS (Method 2) and Urine Analysis

Punches from DUS or HGA solution spots (7-mm-diameter) were transferred to Eppendorf tubes and eluted for 30 min with 250 µL of 10 mmol/L potassium phosphate buffer, pH 5.5, with occasional gentle shaking. Spot eluates were analysed by RP-HPLC as described above. HGA content in DUS was quantified on the basis of concentration/peak area linear plots obtained from eluted spots of HGA solutions of known concentration (prepared as described in Sect. 2.2). Stability of HGA in DUS was checked over 2 weeks storage.

Urine samples from all patients were also directly analysed by the above RP-HPLC method, after heating at 56°C for 30 min and diluting with 30 volumes of 10 mmol/L potassium phosphate buffer, pH 5.5. Urine HGA content was expressed as mmol/L, or mmol/mol creatinine, or, when possible, mmol/24 h.

HGA recovery during 24 h urine storage at 4°C was checked by adding a known amount (final 10 mmol/L) to control urines; storage was conducted both in the absence and presence of acid (final 0.25 mol/L HCl or H_2SO_4).

Results

Colour-Based Semi-Quantitative Method (Method 1)

Both HGA solutions and their dried spots developed brown colour after addition of NaOH. Colour intensity was proportional to HGA concentration from 1.4 (the lowest developing appreciable colour) to 46 mmol/L (Fig. 1a, from 1 to 6). An example of the brown colour developed after alkali addition by five AKU patient DUS is shown in Fig. 1b (1–5); no colour was developed by control (number 6). Concentration values were estimated by comparing colour intensity with HGA solution spots and were expressed as the range between the two standard spots comparable to patient DUS. For each of the ten patients examined, comparison of colour-based values with those determined by Method 2 in eluted spots and by direct HPLC analysis of urines supported the reliability of semi-quantitative evaluation (Table 1).

The developed colour was stable for at least 3 months; afterwards, slight intensity decrease was observed to occur in both DUS and HGA solution spots, still allowing HGA estimation by comparison.

HPLC Analysis: Method Validation

The described HPLC method, initially devised to measure purines, pyrimidines and other metabolites in plasma and urines (Micheli et al. 1999), allows the identification and quantification of several compounds, including creatinine, uric acid, tyrosine, hypoxanthine, xanthine, HGA, phenylalanine and tryptophan (Fig.2a and b).

Concentration/peak area plots developed for HGA quantification exhibited a good linear fit over the range examined (20–800 µmol/L) with $r^2 = 0.999$.

Accuracy was determined in urine matrix. Percentage recovery of a nominal amount of HGA (8–10 mmol/L) spiked into matrix ($n = 6$) was 94.3–110.8%. Imprecision was determined with $n = 6$ using spiked urines (8–10 mmol/L HGA); %CV was 8.7%.

The limit of quantification was 5 µmol/L, the lowest concentration allowing a CV<20%.

HGA recovery during 24 h urine storage at 4°C (checked as described in Sect. 2.5) was 94.3–107.8% in the absence and 102.3–110.8% in the presence of acid.

HPLC Analysis of Urines and DUS (Method 2)

HGA in DUS and in urines was quantified as described under methods (see Sects. 2.4 and 2.5). Concentration/peak area plots obtained from eluted spots of five HGA solutions of known concentration exhibited a good linear fit over the range examined (2.8–23 mmol/L), with $r^2 = 0.995$.

HGA amount measured in DUS from nine patients (14.0 ± 5.2 mmol/L) was in good agreement with that directly measured in their urines (14.7 ± 4.1 mmol/L) with 76.5–117.0% recovery. Reproducibility of results in DUS analysis was tested by repeating the whole procedure on six separate DUS from one patient's urine (CV = 6.9%). Reproducibility test was repeated after 2 week storage of DUS, with no appreciable loss compared to fresh spots (recovery 93.7–105.6%; CV = 8.7%). These data and values expressed as mmol/mol creatinine (1,741 ± 541) or mmol/24 h (17.0 ± 7.4) were in agreement with those reported in the literature (Bory et al. 1989; Mannoni et al. 2004; Hughes et al. 2014; Ranganath et al. 2016). No appreciable HGA could be detected in DUS or urine samples from controls.

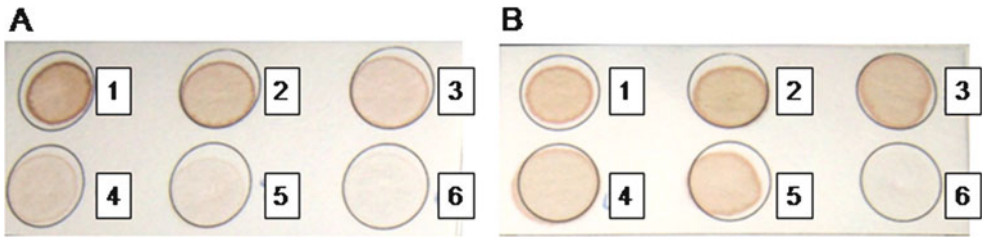

Fig. 1 Colour development in alkali – (**a**) HGA solution spots (from 1 to 6: 46, 23,11.5, 5.8, 2.8, 1.4 mmol/L); (**b**) AKU patient urine spots (from 1 to 5); control urine spot (6). NaOH (1 mol/L 10 μl) was dropped on all spots

Table 1 HGA concentration (mmol/L) measured in DUS and urine of AKU patients. DUS: method 1, colour development (estimated range); method 2: HPLC-linked detection. Urine: direct HPLC-linked detection

Patient	Age	Sex	DUS method 1	DUS method 2	Urine
1	69	F	11.5–23	18.7	15.9
2	50	F	10–20	15.4	16.9
3	41	F	10–20	11.9	13.2
4	48	F	11.5–23	11.9	16.9
5	67	F	10–20	17.0	16.7
6	42	M	11.5–23	19.6	16.0
7	57	M	11.5–23	na	12.6
8	53	M	5–10	4.0	5.3
9	65	M	10–20	18.4	20.3
10	64	M	10–20	9.2	11.4

na not analysed

Results obtained by methods 1 and 2 in DUS, and by direct urine analysis by HPLC are summarized in Table 1.

There was no significant difference in urine HGA concentration between female and male patients (*t* test analysis), nor was there any significant correlation with age in this cohort of patients.

Discussion and Conclusions

Early diagnosis of AKU is not frequently accomplished and at our knowledge only few data are available about HGA concentration in biological fluids of AKU patients before ochronotic symptoms occur. Availability of simple diagnostic methods would allow increased awareness of the disease before irreversible ochronotic arthropathy occurs. Early therapeutic trials to prevent such serious consequences would also be possible.

Biochemical diagnosis is based on the detection of HGA in body fluids, mainly urine, by different analytical methods such as high performance liquid chromatography (Bory et al. 1989), gas chromatography, capillary electrophoresis, mass spectrometry (Hughes et al. 2014), enzymatic and spectrophotometric methods (Tokuhara et al. 2014). Such sensitive methods can only be performed in specialized laboratories with appropriate equipments. In this paper we present two methods developed for the analysis of dried urine spots. Method 1 is a simple method based on colour development by alkali addition on paper dried urine spots (DUS), suitable for HGA approximate quantification. This method does not require any complex equipment and can be used by practitioners or parents, quickly providing a rough evaluation of HGA presence in urines, indicating the range of HGA excretion. Industrial production of alkali pre-soaked papers would render such operation even easier and suitable for the early diagnosis of alkaptonuria. Similar procedures have been reported by other authors as “dipstick test” with a mere qualitative purpose (“yes-or-no” colour development) (Barbas et al. 2002; Zibolen et al. 2000; Koska and Srsen 1977). The novelty in our system is that colour-based quantification on paper DUS can be achieved by comparison with spots of HGA solutions of known concentration, sensitivity reaching 1.4 mmol/L. Such value, the lowest HGA concentration displaying appreciable colour development in dried spots, is tenfold lower than usually found in adult patients; nevertheless such sensitivity might be useful for early detection, e.g., in children. Larger screening in all members of AKU affected families, and early identification of the disease might be achieved by this method, and only selected samples should be conveyed to laboratories for accurate quantification.

Method 2 described in this paper allows the quantification of HGA in DUS by our HPLC-linked method, with results in good agreement with direct urine analysis. Such HPLC-linked method with UV detection is sensitive and reliable and can be performed in many laboratories.

The use of HPLC-linked methods for HGA analysis in body fluids is not new (Bory et al. 1989); the novelty of the proposed method lies in its utilization for DUS, which are easy to collect and deliver, thus representing a low-cost reliable analytical tool for AKU early diagnosis.

Both urine and serum HGA concentration are of paramount importance in the follow up of the disease and in monitoring the effect of drugs. Due to its sensitivity (lower limit 5 μmol/L HGA) the described HPLC method can be applied to any body fluid with low HGA content

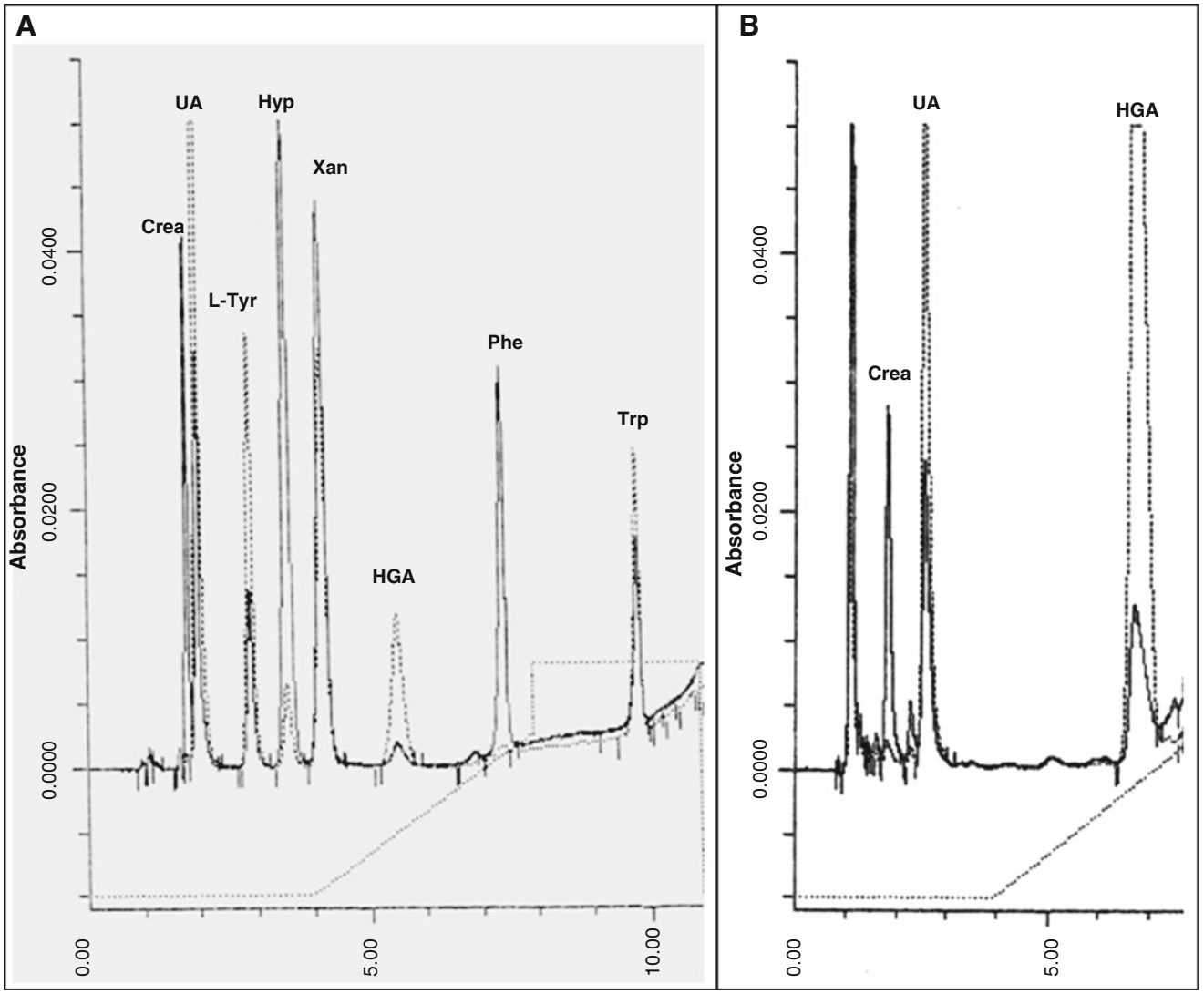

Fig. 2 HPLC profiles of absorbance (*dotted line* 280 nm, *continuous line* 260 nm) – standard mixture of creatinine, uric acid, L-tyrosine, hypoxanthine, xanthine, HGA, phenylalanine and tryptophan (**a**); AKU patient urine (**b**)

such as plasma/serum, which are in the micromolar range (Bory et al. 1989; Hughes et al. 2015).

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Compliance with Ethics Guidelines

Conflict of Interest

Gabriella Jacomelli, Vanna Micheli, Giulia Bernardini, Lia Millucci and Annalisa Santucci declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Contribution of Authors

Gabriella Jacomelli: planning, conducting the experimental part, analyzing and reporting results.

Vanna Micheli: planning, analyzing and reporting results.

Giulia Bernardini : data analysis and reporting results.

Lia Millucci : contacting patients, managing samples and planning experiments.

Annalisa Santucci: planning and supervising.

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